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Mesenchymal stem cells upregulate Treg cells via sHLA-G in SLE patients

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ABSTRACT

Background: Soluble human leukocyte antigen-G (sHLA-G) is a non-classical HLA class I molecule, exhibiting strong immunosuppressive properties by inducing the differentiation of T regulatory cells (Treg). Mesenchymal stem cells (MSCs) transplantation alleviates disease progression in systemic lupus erythematosus (SLE) patients. However, the underlying mechanisms are largely unknown.

Objectives: To explore whether sHLA-G is involved in upregulating effects of MSCs on Treg, which contributes to therapeutic effects of MSCs transplantation in SLE.

Methods: The serum sHLA-G levels of SLE patients and healthy controls were detected by ELISA. The percentages of peripheral blood CD4 + ILT2 +, CD8 + ILT2 +, CD19 + ILT2 + cells and Treg cells were examined by flow cytometry. Ten patients with active SLE, refractory to conventional therapies, were infused with umbilical cord derived MSCs (UC-MSCs) and serum sHLA-G was measured 24 h and 1 month after infusion. The mice were divided into three groups: C57BL/6 mice, B6.MRL-*Fas*^{lpr} mice infused with phosphate buffer saline (PBS), and B6.MRL-*Fas*^{lpr} mice infused with bone marrow MSCs (BM-MSCs). Then, the concentrations of serum Qa-2 were detected. Peripheral blood mononuclear cells (PBMCs) were isolated from SLE patients and co-cultured with UC-MSCs for 3 days at different ratios (50:1, 10:1, and 2:1) with or without HLA-G antibody, and the frequencies of CD4 + CD25 + Foxp3 + T cells were then determined by flow cytometry.

Results: The concentrations of serum sHLA-G were comparable between SLE patients and healthy controls. However, there was a negative correlation between sHLA-G levels and SLE disease activity index (SLEDAI) scores in active SLE patients (SLEDAI > 4). We found that serum sHLA-G levels were negatively correlated with blood urea nitrogen, serum creatinine and 24-hour urine protein in SLE patients. The sHLA-G levels were significantly lower in SLE patients with renal involvement than those without renal involvement. The expression of ILT2 on CD4 + T cells from SLE patients decreased significantly compared to that of healthy controls. A positive correlation between the frequencies of Treg and CD4 + ILT2 + T cells was found in SLE patients. The levels of sHLA-G increased 24 h post UC-MSCs transplantation. The concentrations of Qa-2 in BM-MSCs transplanted mice were significantly higher than those of control group. *In vitro* studies showed that MSCs increased the frequency of Treg cells in SLE patients in a dose-dependent manner, which was partly abrogated by the anti-HLA-G antibody.

Conclusions: Our results suggested that MSCs may alleviate SLE through upregulating Treg cells, which was partly dependent on sHLA-G.

1. Introduction

Systemic lupus erythematous (SLE) is a chronic multisystem autoimmune disease with a broad spectrum of clinical manifestations [1]. SLE is characterized by hyperactivation of B lymphocytes and high titers of serum autoantibodies, Th1/Th2 cell imbalance and tolerogenic regulatory T cells (Treg) reduction [2,3]. Most studies have reported a reduced number and impaired function of Treg cells in active SLE [4]. Low peripheral Treg frequency is a genetically determined predisposing factor for SLE development.

Mesenchymal stem cells (MSCs) are non-hematopoietic progenitors which could differentiate into several mesenchymal tissues, including skeletal tissues, adipose tissues and myocardium [5]. Since 2007, allogeneic umbilical cord derived MSCs transplantation (MSCT) had been used in the treatment of refractory and severe SLE patients in our center [6,7]. Accumulative studies have reported that MSCs can upregulate Treg cells in a dose-dependent manner both *in vitro* and *in vivo* [8,9]. Human leukocyte antigen-G (HLA-G) is a non-classical HLA class-I molecule and exhibits strong immunosuppressive properties, which plays an important role in tumor escape, maternal immune, autoimmune

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diseases and allograft transplantation acceptance [10,11]. Mouse Qa-2 is the functional homolog of HLA-G [12]. Soluble forms of HLA-G (sHLA-G) have attracted much attention and have direct effects in immune inflammation [11]. Studies have found that MSCs can express sHLA-G, which is involved in MSCs-mediated immunoregulation [13,14]. The altered expression of sHLA-G has been reported in the regulation of the pathology of autoimmune diseases [15–17]. However, the levels of sHLA-G in SLE patients, as reported, were inconsistent [3,18–20]. Furthermore, the known receptors for sHLA-G include inhibitory receptors immunoglobulin-like transcript 2 (ILT2) and immunoglobulin-like transcript 4 (ILT4), non-inhibitory receptors CD8 and CD160, and the killer cell immunoglobulin-like receptor (KIR2DL4) [21]. ILT2 is commonly expressed on T cells and some studies have found the defective expression of ILT2 in SLE patients [22–24], which may participate in the defective immunoregulation observed in patients with SLE. It has been reported that there was a significant correlation between sHLA-G and the frequency of Treg cells [15], but the mechanisms are not clear.

As both sHLA-G and MSCs exhibit immunomodulatory functions, we hypothesize that sHLA-G secreted by the infused MSCs regulates T cell differentiation, quantity and even immunosuppressive functions, which ultimately improves disease activity. Therefore, we aim to elucidate the mechanism of MSCs transplantation in SLE treatment and provide a theoretical basis for the broader application of MSCs transplantation in the treatment of lupus.

2. Materials and methods

2.1. Patients and healthy subjects

A total of 68 SLE patients and 32 healthy subjects were included in this study. The patients were diagnosed according to the American College of Rheumatology for classification of SLE. This study was approved by the Ethics Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School and was conducted in accordance with the 1989 Declaration of Helsinki.

2.2. Antibodies and reagents

The following antibodies and their isotype-matched controls (to human) were from eBioscience (San Diego, CA, USA): fluorescein isothiocyanate (FITC)-conjugated anti-human CD4 (OKT3), phycoerythrin (PE)-conjugated anti-human ILT2 (HP-F1), Foxp3 (PCH101), allophycocyanin (APC)-conjugated anti-human CD25 (BC968), CD19 (HIB19), CD8 (RPA-T8). Purified anti-human HLA-G antibodies were from BioLegend (San Diego, CA, USA). The sHLA-G ELISA kit was from USCN (Wuhan, Hubei, China). The mouse Qa-2 ELISA kit was from Shanghai HengYuan Biological Technology Co. (Shanghai, China).

2.3. ELISA assay and clinical data

We detected the levels of sHLA-G and Qa-2 in the conditioned media and/or human serum with ELISA kits according to the manufacturer's instructions. All sample tests were performed in duplicates. Individual clinical data were collected in detail from patients with SLE. SLE activity was assessed by the disease activity index for lupus patients (SLEDAI) [25]. We analyzed the clinical correlations of sHLA-G with SLEDAI scores, disease duration, serum complement 3 (C3) and C4, routine blood test (white blood cells, hemoglobin and platelets), liver function (alanine and aspartate transaminase), kidney function (blood urea nitrogen, serum creatinine, uric acid, 24-hour urine protein), seroimmunological indexes, etc.

2.4. Isolation and culture of MSCs

Fresh umbilical cords were obtained from informed healthy mothers in our hospital after normal deliveries. The umbilical cords were rinsed twice in phosphate buffer saline (PBS), and the cord blood was removed during this process. The washed cords were cut into 1 mm^2 pieces and floated in Dulbecco's modified Eagle's medium (DMEM)/F-12 (Gibco) containing 10% Fetal Bovine Serum (FBS, Gibco) and 1% Penicillin-Streptomycin (Gibco). The pieces of the cord were subsequently incubated at 37 °C in a humidified atmosphere consisting of 5% CO_2. Non-adherent cells were removed by washing with PBS. The medium was replaced every three days after the initial plating. When well-developed colonies of fibroblast-like cells appeared after ten days, the cultures were trypsinized and transferred into a new flask for further expansion.

For acquisition of bone marrow-derived MSCs (BM-MSCs), healthy donors between the ages of 18 and 40 years, who had no history of any significant illness and no physical or mental disability, were selected from members of the patients' family without HLA matching. All gave written informed consent. BM-MSCs were isolated by density gradient centrifugation and adherence methods as described previously with slight modification [26]. Briefly, mononuclear cells were collected by gradient centrifugation and seeded at a density of 1×10^6 cells/cm^2 in DMEM/F-12 containing 10% FBS and 1% Penicillin-Streptomycin. After three days of culture, non-adherent cells were removed and the medium was changed twice weekly thereafter. Once 80% confluence was reached, adherent cells were seeded at a density of 1×10^4/cm^2 for expansion. After two passages, the cells were harvested. The source of mouse MSCs selected in this study was bone marrow from C57/B6 mice. Bone marrow was obtained by flushing the tibias and femurs using PBS. Preparation of mice BM-MSCs was similar with that of human BM-MSCs.

Flow cytometric analysis confirmed the cells expressed CD106, CD105, CD90, CD71, CD44 and CD29, but not CD34, CD14, CD3 or CD45. The capacity of MSCs to differentiate along adipogenic and osteogenic lineages was evaluated as previously described [9]. The cells at passage 3–5 with a purity of >95% were used.

2.5. Isolation and culture of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of SLE patients and healthy controls by density gradient centrifugation on Ficoll. PBMCs were resuspended in PBS containing 1% bovine serum albumin and 0.1% sodium azide. PBMCs were co-cultured with UC-MSCs for three days at different ratios (50:1, 10:1, 2:1) with or without HLA-G antibodies. After three days, cells were harvested and the percentages of CD4 + CD25 + Foxp3 +, CD4 + ILT2 +, CD8 + ILT2 + and CD19 + ILT2 + cells were examined by flow cytometry.

2.6. Flow cytometry analysis

PBMCs were resuspended in PBS containing 1% bovine serum albumin and 0.1% sodium azide. For the staining of surface antigens, cells were incubated on ice for 30 min with FITC-, PE-, APC-conjugated monoclonal antibodies, or negative control antibodies as indicated. Intracellular staining of Foxp3 was performed.

2.7. Quantitative real-time polymerase chain reaction

Complimentary DNA (cDNA) was synthesized from Trizol isolated total RNA by use of the SuperScript III First-Strand Synthesis SuperMix for quantitative reverse-transcribed polymerase chain reaction (qRT-PCR, Takara). For real-time PCR experiments, reactions containing the SYBR Premix EX Taq (Takara Dalian, China), ROX Reference Dye (503, Takara), cDNA, and gene primers were run on the StepOne Plus Real-Time PCR Systems and analyzed with StepOne Software V2.1 (Applied Biosystems New York, USA). Gene primers were listed in Table 1. The relative gene quantification was done using the $2^{-\Delta\Delta Ct}$ method following normalization to glyceraldehyde-3-phosphate dehydrogenase.

Table 1
Primers for real-time PCR.

Gene	Forward	Reverse
HLA-G	5′-GCCATCGATTTAATTAATACGACTCACTATAG-3′	5′-ATCTAATACCGGTGAATTCCGTTGAGACCTA-3′
GAPDH	5′-GCACCGTCAAGGCTGAGAAC-3′	5′-TGGTGAAGACGCCAGTGGA-3′

Table 2
Demographic features of SLE patients ($n = 80$) and healthy controls ($n = 32$).

	SLE patients	Healthy controls
	$n = 68$	$n = 32$
Age (years, mean ± SD)	35.6 ± 14.5	33.7 ± 8.3
Sex (F/M)	61/7	27/5
Disease duration (years)	5.9 ± 6.6	
SLEDAI (Mean ± SD)	7.8 ± 5.2	
SLEDAI ≤ 4	23 (34%)	
SLEDAI ≥ 4	45 (66%)	

2.8. MSCs transplantation

A total of 10 patients received once intravenous infusion of 1×10^6 UC-MSCs per kilogram of bodyweight. Before transplantation, the cells were washed with PBS to remove FBS from the culture medium. MSCs were slowly infused by a heparinized syringe through the cubital vein of the arm over 30 min. The patients were discharged after at least twenty-four hours of observation.

Eighteen-week-old female C57BL/6 and B6.MRL-*Fas*^{lpr} mice were purchased from Model Animal Research Center of Nanjing University. Ten B6.MRL-*Fas*^{lpr} mice were injected with 0.2 ml of PBS via tail vein, and another ten B6.MRL-*Fas*^{lpr} mice injected with 5×10^5 BM-MSCs at third passage in overall 0.2 ml of PBS medium. Ten C57BL/6 mice were used as the control group. Eight weeks after infusion, mice were sacrificed with CO_2 and blood was taken by heart puncture.

2.9. Statistical analysis

Data were presented as mean ± SEM and analyzed by Student's *t*-test. The correlations were analyzed by Spearman's correlation test. We performed statistical analysis with SPSS16.0 software or GraphPad Prism 5 and a P value of <0.05 was considered as significant.

3. Results

3.1. Serum sHLA-G levels are correlated with creatinine in SLE patients

The serum levels of sHLA-G were measured by ELISA in 68 SLE patients and 32 healthy controls. Demographic characteristics were

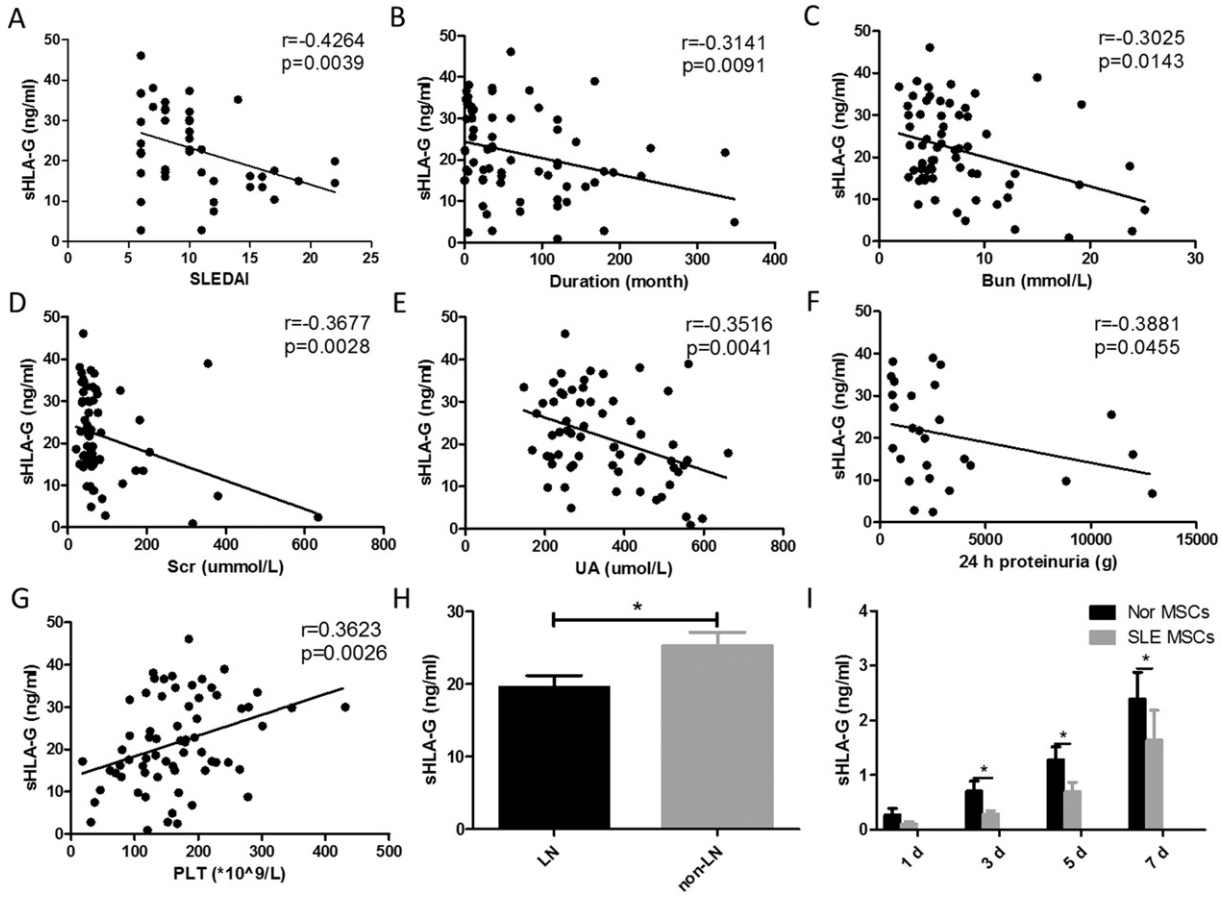

Fig. 1. sHLA-G and clinical parameters in SLE patients. A, correlation between sHLA-G levels and SLEDAI scores in active SLE patients (SLEDAI > 4); B–G, correlation between sHLA-G levels and duration, blood urea nitrogen, serum creatinine, uric acid, 24-hour urine protein and platelet levels; H, the levels of sHLA-G in SLE patients with or without renal involvement; I, the ability of healthy control derived MSCs and SLE patients derived MSCs to secrete sHLA-G. (LN, patients with lupus nephritis; non-LN, patients without lupus nephritis; Nor MSCs, healthy control derived MSCs; SLE MSCs, SLE patients derived MSCs; *, $P < 0.05$).

shown in Table 2. The serum concentrations of sHLA-G were comparable between SLE patients and healthy controls (21.53 ± 10.53 ng/ml *vs* 17.44 ± 9.69 ng/ml, $P > 0.05$). We divided SLE patients into two groups either active or inactive disease. We found a negative correlation between sHLA-G levels and SLEDAI scores in active SLE patients (SLEDAI > 4), indicating that sHLA-G may play a protective role in SLE (Fig. 1A).

To define clinical effects of sHLA-G in lupus, we analyzed the correlations between sHLA-G levels and the clinical parameters. We found that sHLA-G levels were negatively correlated with duration of the disease, levels of blood urea nitrogen, serum creatinine, uric acid, and 24-hour urine protein (Fig. 1B, C, D, E, F). The sHLA-G levels were positively correlated with platelet levels (Fig. 1G). Furthermore, we found that the sHLA-G levels were significantly lower in SLE patients with renal involvement than those without renal involvement (Fig. 1H). There were no correlations between sHLA-G levels and other clinical parameters. It seemed that sHLA-G displayed a protective effect in lupus patients, particularly those with lupus nephritis. In addition, we found

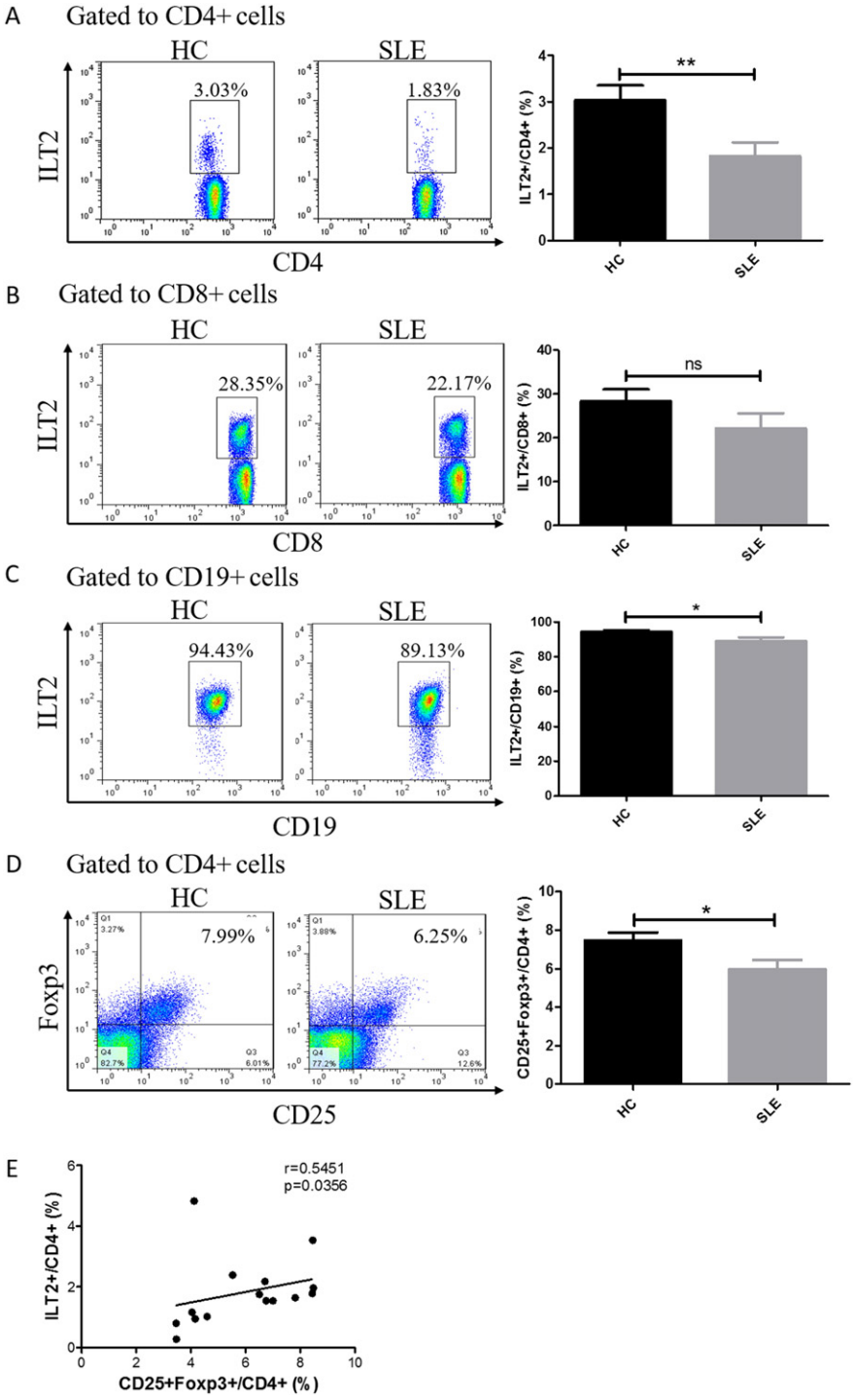

Fig. 2. ILT2 expression on T and B lymphocytes decreased in SLE patients. Peripheral blood mononuclear cells from SLE patients and healthy controls were stained with anti-ILT2-PE, anti-CD4-FITC, anti-CD8-APC, anti-CD19-APC, anti-CD25-APC or anti-Foxp3-PE antibodies and analyzed by flow cytometry. A–C, ILT2 expression on different lymphocyte subsets in patients with SLE and healthy controls. D, Percentages of Treg cells in SLE patients and healthy controls. E, the correlation between Treg cells and CD4 + ILT2 + T cells. (ns, no significant difference; *, $P < 0.05$; **, $P < 0.01$).

that the BM-MSCs from SLE patients secreted lower levels of sHLA-G than those from healthy controls (Fig. 1I).

3.2. ILT2 expression on T and B lymphocytes was downregulated in SLE patients

ILT2 is one of the inhibitory receptors of sHLA-G. Peripheral blood samples from 15 patients and 15 healthy controls were collected to determine the expression of ILT2 on T and B lymphocytes by flow cytometry. The expression of ILT2 on CD4+, CD8+ and CD19+ lymphocytes was analyzed. It showed a significant decreased expression of ILT2 on CD4+ T cells and CD19+ B cells from SLE patients compared to those of healthy controls (Fig. 2A, C). However, the expression of ILT2 on CD8+ T cells was not different between SLE patients and healthy controls (Fig. 2B).

It has been reported that there was a significant correlation between sHLA-G and the frequency of Treg cells [15]. We detected the frequency of Treg cells in the above subjects. Our data showed a significant decreased percentage of Treg cells in SLE patients compared with that of healthy controls (Fig. 2D), which was similar to previous reports [27]. There was no relationship between sHLA-G levels and Treg cells frequencies in SLE patients. However, a positive relationship between the frequencies of Treg cells and CD4+ILT2+ T cells in SLE patients was found (Fig. 2E).

3.3. Serum sHLA-G and mouse Qa-2 increased after MSCs transplantation

We detected the levels of serum sHLA-G in ten SLE patients before and after UC-MSCs transplantation. The results showed that the levels of sHLA-G significantly increased 24 h after MSCT (Fig. 3A). After one month, the levels of sHLA-G decreased in comparison with those 24 h after MSCT, but still showed an increase in comparison with those prior to transplantation.

Qa-2 is the functional homolog of HLA-G in mice. We further evaluated the effect of MSCT on Qa-2 in lupus mice. We used ten normal C57BL/6 mice, ten B6.MRL-*Fas*^{lpr} mice receiving PBS, and ten receiving BM-MSCs transplantation. Eight weeks post BM-MSCs transplantation, the concentrations of Qa-2 in BM-MSCs transplanted mice were significantly higher than those of lupus mice and C57BL/6 mice (Fig. 3B).

3.4. MSCs upregulated Treg cells via HLA-G/ILT2

PBMCs were isolated from SLE patients and co-cultured with UC-MSCs at ratios of 50:1, 10:1 and 2:1. After 3 days of co-culture, we measured the frequency of CD4+CD25+Foxp3+ cells. Our results showed that UC-MSCs upregulated the percentage of Treg cells (Fig. 4A–B). The HLA-G mRNA level in UC-MSCs was significantly increased after co-culture with PBMCs from SLE patients (Fig. 4D). With the treatment of neutralizing antibodies targeting HLA-G in co-culture conditions, the percentage of Treg cells was significantly decreased (Fig. 4A, C). The results also showed that the expression of ILT2 on CD4+T cell was increased (Fig. 4E). These data indicate that sHLA-G, secreted by UC-MSCs, can upregulate the proportion of Treg cells.

4. Discussion

sHLA-G is a soluble isoform of HLA-G and plays a vital role in immune regulation. In autoimmune diseases, sHLA-G has been suggested to play a protection role against autoimmune responses by downregulating inflammatory processes and inducing immune tolerance [28]. Previous studies on sHLA-G levels in SLE patients are conflicting. Rizzo et al. reported that the plasma levels of sHLA-G significantly decreased in SLE patients compared with those of healthy controls [19]. However, Rosado *et al.* and F-X Wu *et al.* found higher concentrations of sHLA-G in SLE patients [18,20]. Some studies failed to show a significant difference of serum sHLA-G levels in SLE patients *versus* healthy controls [3]. In the present study, the concentrations of sHLA-G did not differ significantly between SLE patients and healthy controls. The conflicting results above may ascribe to the complicated conditions of SLE patients. Our results show that the levels of sHLA-G were negatively correlated with duration of disease. The median duration of SLE patients enrolled in Rizzo's study was 9.8 years, while it was 6 years in our study and 3 years in F-X Wu's study. The longer duration may be one of the reasons which resulted in the lower levels of sHLA-G in SLE patients enrolled in Rizzo's study. In addition, two meta-analyses demonstrate that the HLA-G 14 bp insertion allele might act as predisposing factor for SLE, which has been known to be associated with lower expression of sHLA-G [29,30]. A recent study also found that the HLA-G 14 bp insertion allele represented a genetic contribution to early-onset SLE [31]. Thus, we suppose that the abnormal expression of HLA-G gene might result in

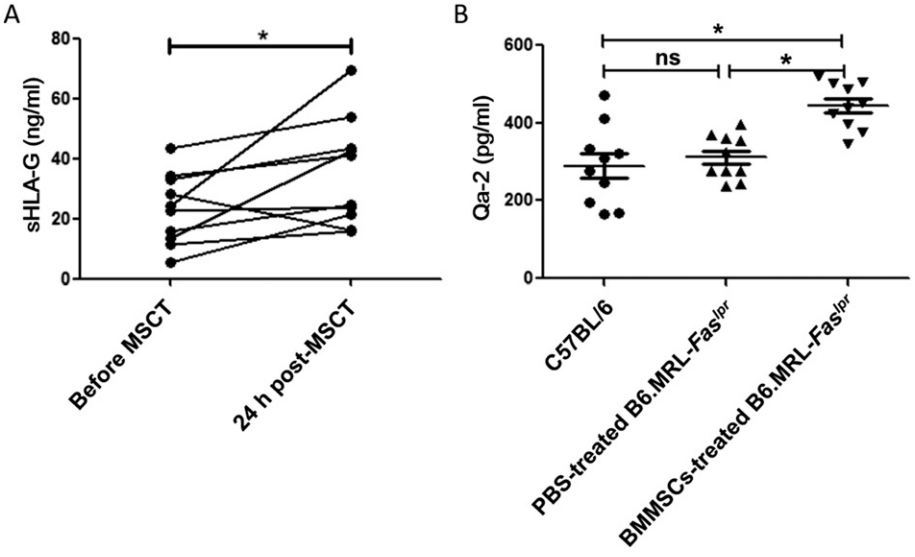

Fig. 3. sHLA-G and mouse Qa-2 increased after MSCs transplantation. Ten SLE patients received once intravenous infusion of 1×10^6 UC-MSCs per kilogram of bodyweight, and the levels of sHLA-G in serum of SLE patients were detected by ELISA. Using ten C57BL/6 mice, ten B6.MRL-*Fas*^{lpr} mice receiving PBS, and ten receiving BM-MSCs, the concentrations of Qa-2 in serum of mice were detected by ELISA. A, the levels of sHLA-G in serum of SLE patients were detected before and 24 h post-MSCT. B, the levels of Qa-2 in mice from different groups. (*, $P < 0.05$).

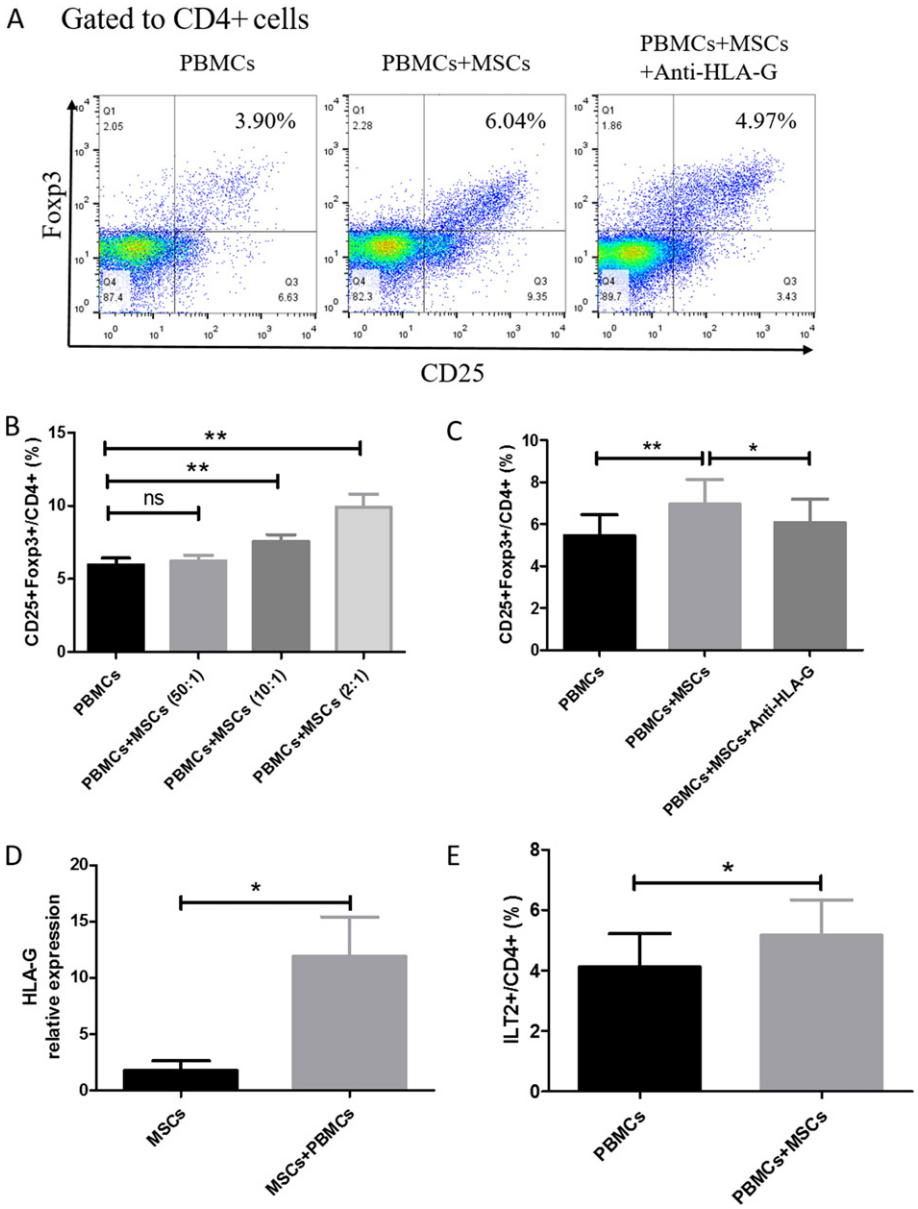

Fig. 4. Immunomodulatory function of UC-MSCs on Treg cells. A, the effect of UC-MSCs on Treg cells proportion with or without anti-HLA-G. B, percentages of CD25 + Foxp3 + cells in CD4 + T cells (Treg) were significantly upregulated by UC-MSCs in a dose dependent manner ($n = 4$ in each group). C, percentages of CD25 + Foxp3 + cells in CD4 + T cells (Treg) were significantly decreased by adding an anti-HLA-G antibody ($n = 4$ in each group). D, the HLA-G mRNA expression in UC-MSCs. E, the expression of CD4 + ILT2 + cells was upregulated by UC-MSCs. (ns, no significant difference; *, $P < 0.05$; **, $P < 0.01$).

altered sHLA-G expression in SLE patients, contributing to the pathogenesis of SLE.

SLE patients were divided into two groups, active and inactive, depending on SLEDAI scores. We found that the levels of sHLA-G were negatively correlated with SLEDAI in the active group. To understand the role of sHLA-G in SLE, we analyzed the associations between clinical parameters and sHLA-G. We found that the sHLA-G levels were lower in patients with lupus nephritis and were negatively correlated with levels of blood urea nitrogen and creatinine. In addition, we found that sHLA-G levels had an inverse relation with 24-hour urine protein in SLE patients with positive urinary protein. Thus, we thought that sHLA-G might be a protective molecule of the kidney. Coincidentally, the reduction of renal inflammation by sHLA-G has been evaluated in a mouse model of lupus nephritis, indicating that sHLA-G might be a vital molecule in SLE patients with renal involvement [32]. As sHLA-G may play a positive role in SLE, it should further be investigated why the expression of sHLA-G increased in SLE patients in some studies. We hypothesized that its function was impaired in SLE patients. The receptors for sHLA-G have been demonstrated to include ILT2, ILT4, CD85d, KIR2L4D and others. Several previous studies on the altered expression of ILT2 in SLE patients have been reported but were conflicting [3,23,24]. We then analyzed the expression of ILT2 on T and B lymphocytes from SLE patients and healthy controls. We found an impaired expression of ILT2 in SLE patients. In fact, the functions of sHLA-G receptor ILT2 on T cells were compared to those of CTLA4 and found to be very similar [21]. Additionally, sHLA-G can upregulate the expression of its receptors [11] and thus may be an effective target of SLE therapy. In line with other authors [33,34], we found a decreased frequency of Treg cells in SLE patients. Many studies have reported the relationship between sHLA-G and Treg cells in several diseases [15,35], but was not found in SLE patients of our study. This indicates that some other factors may influence the frequency of Treg cells.

It has been reported that mesenchymal stem cells can secrete sHLA-G [13,36]. Our study showed that the capability of secreting sHLA-G of

BM-MSCs from SLE patients was impaired compared with BM-MSCs from healthy controls. Soluble HLA-G has been proven to be involved in MSCs-mediated immunoregulation, although the mechanisms remain obscure [10]. We have found that serum levels of sHLA-G increased significantly after transplantation in patients. In the mouse model, eight weeks post BM-MSCs transplantation, the concentrations of Qa-2 in BM-MSCs transplanted mice were significantly higher than that in mice receiving no therapy and normal controls. Allogeneic MSCs transplantation resulted in great therapeutic effects, both in the treatment of SLE patients and mouse models in our center [6,37], whereas further research is required to explore the underlying mechanisms.

MSCs can secrete sHLA-G5 *in vitro*, which has been reported to contribute to the expansion of CD4 + CD25 high Foxp3 + regulatory T cells in mixed lymphocyte reaction [13]. We co-cultured MSCs with PBMCs from SLE patients for three days and found that MSCs can upregulate the frequency of Treg cells in a dose-dependent manner, which was partly abrogated by an HLA-G blocking antibody. The results also showed that the expression of ILT2 on CD4 + T cell increased, indicating that MSCs may upregulate Treg cells via sHLA-G/ILT2. However, further research is needed to understand how MSCs upregulate Treg cells via sHLA-G.

In conclusion, sHLA-G might play a beneficial role in SLE patients, especially in attenuating renal inflammation. MSCs transplantation may alleviate SLE through sHLA-G mediated upregulation of Treg cells proportion.

Conflict of interest

None.

Acknowledgments

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